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PHERMONAL CONTROL OF BITING MIDGES (CULICOIDES SPP).(U)
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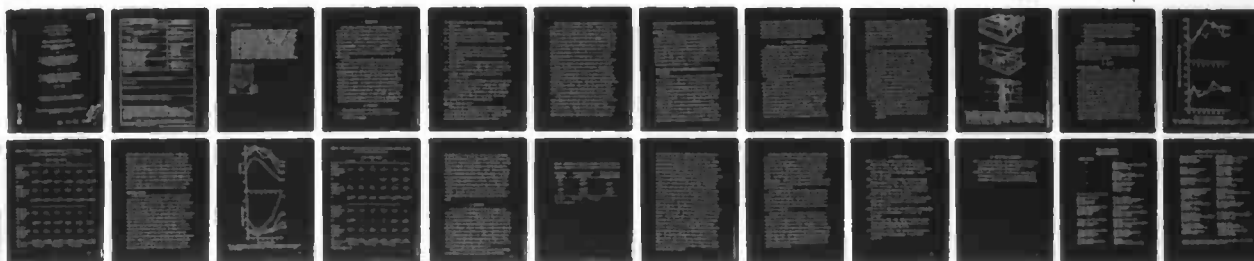
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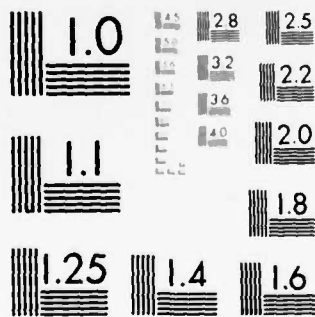
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PHEROMONAL CONTROL OF BITING MIDGES
(CULICOIDES SPP.)

By

Dr. J. R. Linley, co-principal investigator,
Florida Medical Entomology Laboratory,
Institute of Food and Agricultural Sciences,
University of Florida, P. O. Box 520,
Vero Beach, Florida 32960

and

Dr. D. A. Carlson, co-principal investigator,
Adj. Associate Professor, Dept. of Entomology
and Nematology, Institute of Food and
Agricultural Sciences, University of Florida,
Gainesville, Florida 32611

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Abstract continuation

→ The capacity of female Culicoides melleus to elicit sexual response from males was examined in relation to the age of the female. In combination with the age effect, two other factors were tested: whether the female was alive or dead (killed by freezing for 30 min immediately prior to use), and whether the female's wings were present or absent. Male response increased from the time of female emergence up to 2 days. Stimulation was at its greatest in females approximately 1-3 days old. From 3-5 days, response levels declined. Live females, whether with or without wings, were more stimulating than dead ones, and females with wings, whether alive or dead, produced more response than those without. The condition of the female lacking wings seemed, generally, to reduce male response more than the condition of the female being dead. The increase in male response over the first 2 days of female life was interpreted as a mechanism to offset unreceptive behavior in virgin females, which intensifies over the same period, and which otherwise might result in failure of some females to become inseminated.

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I. INTRODUCTION

Biting midges, principally of the genus Culicoides, are important as vectors of human and animal diseases. Particularly, in the last decade, they have gained significance in relation to human health with the occurrence of numerous epidemics of Oropouche virus infection in the Amazon basin. The virus is transmitted in the urban cycle by Culicoides paraensis. As pests, also, biting ceratopogonids are a great hindrance to coastal recreation, development and industry, and they cause annoyance and reduce efficiency at coastal military installations.

A deficiency in research with these insects over the past 20 years has been the failure to concentrate effort in fundamental investigations of biology and behavior. When an analysis of the sexual behavior of Culicoides melleus was undertaken, the males were found to respond to the females on contact, and subsequent investigation revealed the presence of a relatively non-volatile contact pheromone, which could be extracted in hexane. The purpose of the research program here reported was to search for the existence of both attractant and contact pheromones that mediate sexual behavior in Culicoides, with a view to possible modification of behavior for control effect. Emphasis was placed on Culicoides melleus and also, initially, C. variipennis since this species could be held relatively easily in laboratory culture.

II. OBJECTIVES

The principal objectives of the study were as follows.

- (1) Confirmation of bio-assay as valid method of investigation of stimulants for C. melleus.

- (2) Specific chemical identification of the C. melleus contact pheromone(s).
- (3) Synthesis of the C. melleus pheromone(s).
- (4) Bio-assay of synthesized materials and investigations of possible synergistic effects if more than one stimulant is involved.
- (5) Structure-activity relationships using synthesized stimulant compounds.
- (6) Detailed investigation of the exact role of the contact pheromone(s) in the sequence of sexual behavior, including examination of whether pheromone gradients on the female body provide cues for male orientation.
- (7) Investigation of effects of melleus pheromone on the behavior of other species, especially C. furens, a major pest in coastal areas.
- (8) Examination of cuticular hydrocarbons of other species, initially - C. furens, C. variipennis; assay and identification of sex pheromones if present.
- (9) Use of already constructed olfactometers in the search for possible attractant substances, initially in C. melleus and C. variipennis.

The objectives divide, essentially, into 2 groups which may be considered under the headings, respectively, of chemistry (objectives 2, 3, 4, 5) and behavior (objectives 1, 6, 7, 8, 9). Not all have been addressed as the amount of work is prohibitive.

Chemistry - Objectives 2 and 3

The aim has been to fractionate the crude hexane extract from female C. melleus and C. variipennis and subject the compounds obtained to further analysis designed to reveal structure. The methods used have been conventional and need not be described in detail.

Crude extracts of C. melleus and C. variipennis have been subjected to chemical fractionation on silica-gel columns. Analysis by gas chromatography has shown the presence of essentially the same materials in hydrocarbons from both sexes from frozen samples of C. variipennis from the Denver colony. Mass spectra of collected C. melleus paraffins after separation from non-paraffins showed that the methyl-branched paraffins with 35 and 37 carbon chains had methyl branches at primarily the 11-, 13-, or 17- positions. Dimethyl-branched paraffins with 35, 37 and 39 carbon chains had methyl branches at the 11, X or 13, X- positions, apparently separated by 7 or 9 methylene units. Earlier eluting compounds of 21 to 33 carbons were unbranched paraffins in both sexes. All of the above materials have been quantitated for both sexes in C. melleus. Hydrocarbon materials in laboratory C. variipennis have a similar composition to those in melleus, with unbranched paraffins predominating in compounds with less than 33 carbons, and with mono- and dimethyl branched compounds having odd numbers of carbons in their backbones at 35, 37 and 39 carbons. These materials have been submitted for mass-spectral analysis. Also, several polar materials of more than 400 molecular weight were eluted from the silica-gel columns and analysed by gas chromatography, but have not yet been characterized.

Further progress in the identification of the very many hydrocarbon compounds encountered will probably require extraction, fractionation and analysis of additional material from groups of many thousands of insects. Completion of the mass-spectral interpretation will also be necessary. The mass spectra are partly analysed at the present time and we defer reporting further on this segment of the

work until these interpretations have been brought to a more intelligible stage.

Behavior - Objective 6

Hexane extractions already done (Linley and Carlson, 1978) had succeeded in removing many hydrocarbon compounds, including those with biological activity, from the female cuticle of Culicoides melleus. However, there was no basis for knowing during what period, from the time of emergence, pheromone qualities were at a maximum. Thus, we tested whether females of different ages after emergence were equally stimulating to males. Two other factors of biological interest were also examined, as discussed below. The rationale, methods and results are described and discussed in detail as follows.

Sexual response of males to C. melleus females of different ages after emergence.

We assayed male response to females in 6 age-groups in combination with 2 other factors. The first of these factors was necessary to isolate the effect of female age. Linley & Adams (1974) showed that receptivity of virgin female melleus decreases with age. Females increasingly display resistive behaviors (towards the male) that were thought likely to affect results from methods selected in this study to assess male response. In one experimental group, therefore, the females were dead, having been killed immediately prior to use. Secondly, we wished to determine whether male behavior was affected by the presence of the female's wings. Culicoides melleus mates without flight and the wings of the female are folded and closely cover much of the top of the abdomen (Linley & Adams 1972). Sexual stimulation of the male is very probably through tarsal receptors, since at

contact with a female, males immediately clamber entirely onto the female's body and achieve sexual orientation without touching the supporting surface. It is inevitable that much tarsal-wing contact occurs and it was of interest to evaluate the effect of wings on male response and behavior during precopulatory orientation.

III. MATERIALS AND METHODS

Culicoides melleus males and females were collected as pupae from natural breeding sites, sexed prior to emergence, and induced to emerge as before (Linley & Adams 1972). Both males and females were kept individually, with access to 10% sucrose, until used, as virgins. Females were of the ages specified and all males were 12-24 h old. Females were killed by freezing at -20°C for 30 min just before use. When required, the female wings were removed very close to the point of attachment to the thorax. All observations were done at $25-27^{\circ}\text{C}$ in round Plexiglas cells (Fig. 1).

Insects were confined for observation as in previous behavioral work (Linley & Adams 1972). In addition, however, the confinement cell was adapted to a device which permitted the dead female to be mounted on a minuten pin and rotated around the cell in a position adjacent to the cell wall, onto which the males invariably clambered. Thus, dead females could be presented with first male contact at the anterior or posterior end of the female, as desired. A dead female was oriented on the minuten so that she assumed a position as if walking on the cell wall, as did live females and males. Mounting pins were bent at the tip and inserted into the lower thorax so that the pin's shaft did not obstruct males clambering atop the female body

during precopulatory orientation. It was assumed that males would make first contact with live females at each end with about equal frequency. Dead females were presented from each direction alternately. Insects were introduced into the cells without anesthesia and were kept apart until desired by a stainless steel slider, which partitioned the observation cell. The complete device may be of some general utility for behavioral work with small insects and is described in Fig. 1.

The females tested were in 6 age-groups after emergence; 1-2 h, 1 day, 2 days, 3.5 days, 5 days and 7 days. For each group, there were 4 conditions of the female: live with wings-L (ww) and no wings-L (nw); dead with wings-D (ww) and no wings-D (nw). A test was conducted as follows, beginning 3 min after insects had been introduced into the apparatus. Each male was allowed 5 attempts, in immediate succession, to achieve copulation. The interval between attempts could not be regulated with live females; with dead females, 10 sec were allowed between attempts. No test exceeded 10 min total duration. The following data were recorded: a) total duration of attempt, resolved to 0.2 sec with an event recorder (Evans 1975), from first contact to either loss of contact or attainment of sexual union (see Linley & Adams 1972); b) whether or not copulation was achieved and; c) a response score scaled from 1 to 5 according to the following criteria.

- 1 - Male shows full sexual response and achieves sexual union.
- 2 - Male shows intense excitement, rotates terminalia, opens claspers, orients to the rear of the female but does not sustain the copulatory position, and does not unite the genitalia.

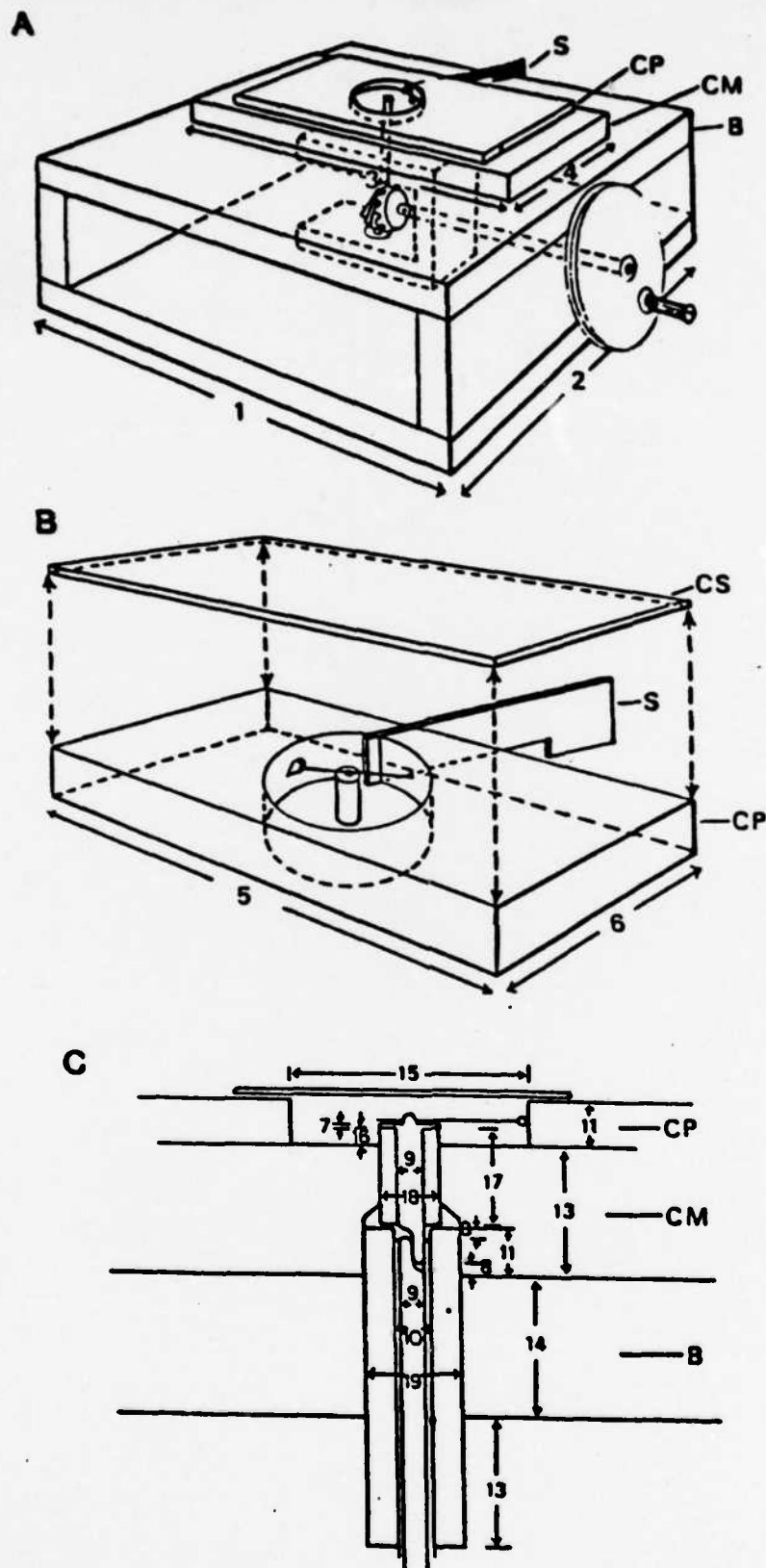


Fig. 1. Apparatus used for behavioral assay. A, entire device; B, detail of confinement cell; C, detailed vertical section through rotating, removable minuten mount and drive shaft. All parts of Plexiglas except stainless steel slider, glass coverslip and brass minuten mount, gears and drive shafts. Key - S, slider; CP, cell piece; CM, cell mount; B, base; CS, coverslip; 1 and 2 = 13.0 cm; 3 = 10.0 cm; 4 = 3.9 cm; 5 = 7.7 cm; 6 = 3.1 cm; 7 = 0.4 mm; 8 = 0.8 mm; 9 = 1.6 mm; 10 = 2.3 mm; 11 = 3.2 mm; 12 = 6.7 mm; 13 = 8.7 mm; 14 = 9.6 mm.

- 3 - Male shows pronounced excitement and quickened movements associated with sexual response, rotates terminalia and opens claspers, but does not achieve sustained orientation.
- 4 - Male shows discernible excitement, but no movements of terminalia.
- 5 - Male shows no response.

An understanding of the behaviors will be facilitated by reference to Linley & Adams (1972). Results from 60 pairs of insects for each age and condition were obtained. All the χ^2 analyses were performed on data before conversion to percentages.

IV. RESULTS

Duration of male attempt

Among data for attempt duration, only the category 1 responses (copulation achieved) are considered since the other categories represented incomplete responses and were of more limited interest. Attempts on live females (Fig. 2, upper) followed very closely the course expected from earlier work (Linley & Adams 1974), in which virgin females displayed increasingly intense unreceptive behavior as they grew older. Males took progressively longer to achieve sexual union as female age increased, especially through the first 3.5 days. It is during this period that pronounced increase in the resistive behavior of females take place (Linley & Adams 1974). The presence or absence of wings produced no consistent, clear effect. In dead females, with unreceptive behavior eliminated as a factor (Fig. 2, lower), there was indication of an increase in male attempt time on progressively older females, suggesting that older females were less

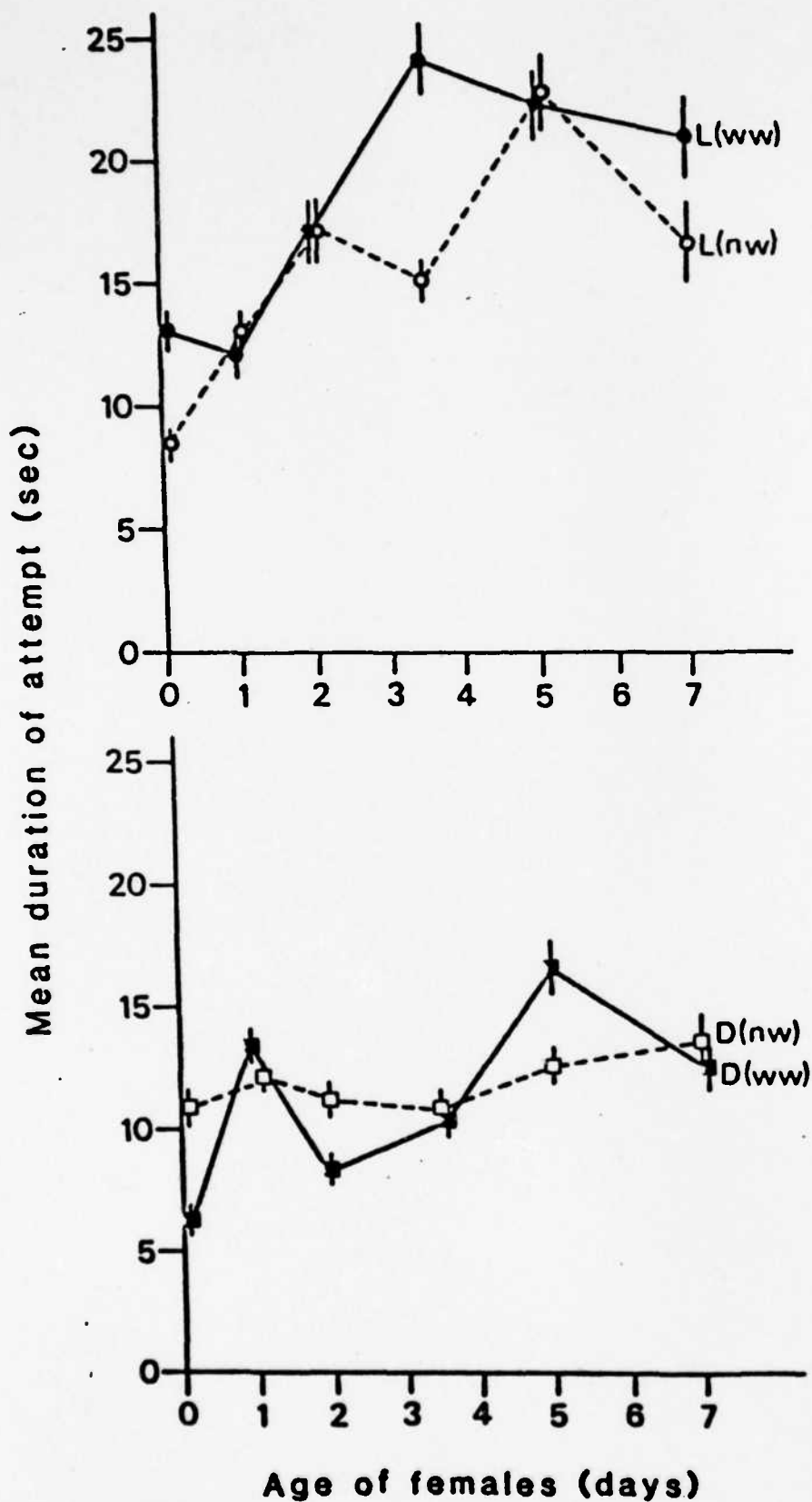


Fig. 2. Relationship of duration of male attempt to female age. L, live; D, dead; ww, with wings, nw, no wings. Points show means of standard errors.

stimulating to males. The effect, however, was not pronounced. There was no difference clearly related to the presence or absence of the female's wings.

Intensity of male response

A better measure of male behavior was obtained from the response scores, as these were not subject to variation arising from differences in the speed with which individual males achieve orientation on the female. Although scores were assigned in unit increments, we do not imply exact correlation with actual stimulus levels, which remain unknown. The data are used in two ways; in one the average response score is shown (Fig. 3, upper) and, in the other, the combined proportions of responses in categories 1 + 2 (summed) are shown (Fig. 3, lower). These scores were combined to avoid low expected numbers in χ^2 analysis and because both involved complete preparatory movements of the genitalia and orientation on the female. Response 2 differed from 1 only in the failure of the male to sustain the copulatory position and unite the genitalia.

Similar conclusions are indicated by both treatments of the data. An analysis of the proportions of attempts in response levels 1 + 2 showed every group to be heterogeneous with respect to female age (χ^2 for L (ww) = 32,046, L (nw) = 55.506, D (ww) = 23,368, D (nw) = 34.775, with $P < 0.001$ in all instances). The females increased in their capacity to stimulate males during the first 2 days after emergence, whereafter response declined to levels somewhat but not greatly lower than at emergence. The period of maximum stimulation was from about 1-3 days. Live females were more stimulating than

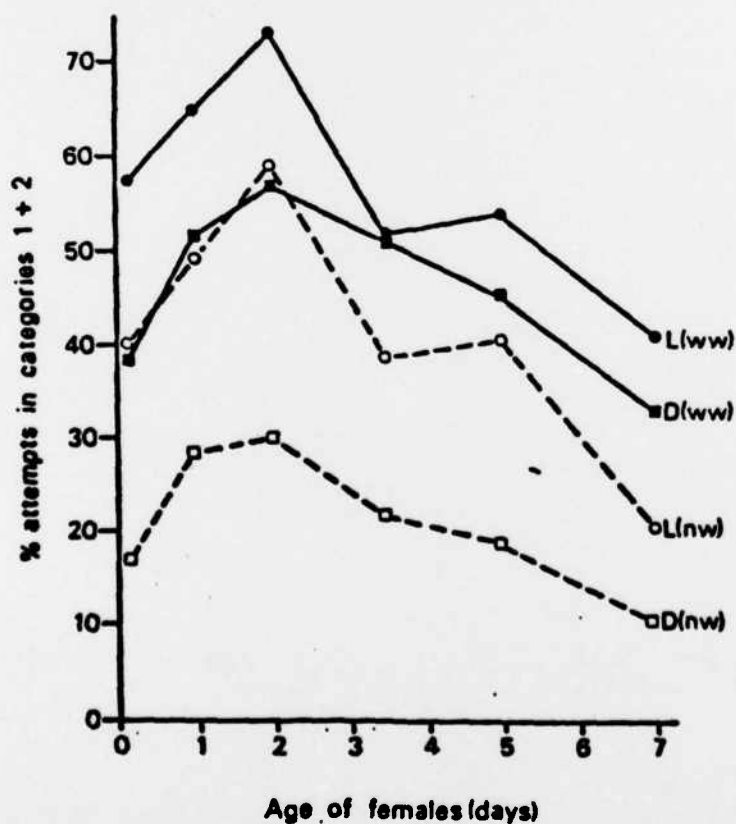
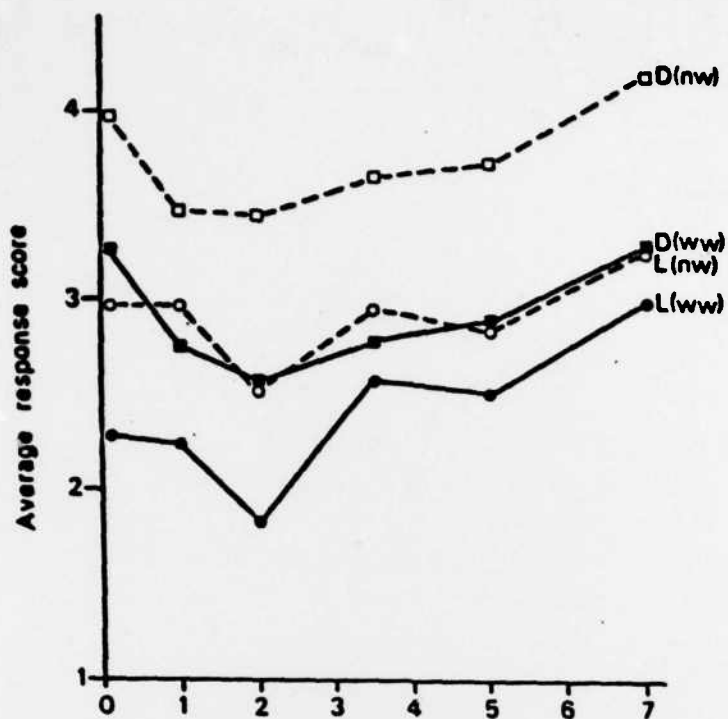


Fig. 3. Relationship of male response to female age. L, live; D, dead; ww, with wings; nw no wings.

TABLE 1. Proportion of male responses in categories 1 + 2 as related to presence or absence of wings and living or dead state of females.

	Age of females (days)					
	1-2 (hr)	1	2	3,5	5	7
<u>Live ♀♀</u>						
Difference in % (with wings - no wings)	17.3	21.2	14.5	13.2	13.0	20.4
χ^2	7.368 ⁺⁺⁺	10.902 ⁺⁺⁺⁺	4.601 ⁺	5.564 ⁺⁺	5.171 ⁺	21.191 ⁺⁺⁺⁺
<u>Dead ♀♀</u>						
Difference in % (with wings - no wings)	21.5	23.2	27.2	29.3	26.8	22.0
χ^2	20.670 ⁺⁺⁺⁺	17.672 ⁺⁺⁺⁺	19.745 ⁺⁺⁺⁺	27.009 ⁺⁺⁺⁺	30.202 ⁺⁺⁺⁺	31.848 ⁺⁺⁺⁺
<u>With wings</u>						
Difference in % (live ♀♀ - dead ♀♀)	18.8	13.1	16.4	0.9	8.5	8.4
χ^2	8.840 ⁺⁺	4.021 ⁺	5.557 ⁺⁺	0.000	2.119	2.719
<u>No wings</u>						
Difference in % (live ♀♀ - dead ♀♀)	23.0	15.1	29.1	16.2	22.2	10.0
χ^2	23.143 ⁺⁺⁺⁺	8.365 ⁺⁺⁺	24.187 ⁺⁺⁺⁺	11.522 ⁺⁺⁺⁺	22.229 ⁺⁺⁺⁺	9.527 ⁺⁺⁺
⁺ P < 0.05	⁺⁺ P < 0.02	⁺⁺⁺ P < 0.01	⁺⁺⁺⁺ P < 0.001			

dead and females with wings more than those without. A comparison between pairs of proportions at each age (Table 1) shows that these differences were almost all significant. With live females, those with wings consistently elicited a higher proportion of combined 1 + 2 responses and all differences were significant. Similar, but generally larger and more highly significant differences were obtained with all groups of dead females (Table 1). In contrasts between females with and without wings, live individuals uniformly produced greater response, with the difference significant in the youngest 3 groups with wings, and all differences highly significant among groups with no wings.

Copulations achieved

If the attainment of copulation is examined as a measure of male response (Fig. 4), the same conclusions emerge. The proportions of males that succeeded in copulating (Fig. 4, upper) change significantly with age within each group (χ^2 for L (ww) = 52.352, L (nw) = 51.092, D (ww) = 25.429, D (nw) = 38.399, with $P < 0.001$ in all cases). Between group comparisons (Table 2) showed that females with wings were more stimulating to males and a greater proportion was mated in every case. The differences for live females, where comparisons were permissible, were not significant, but in dead females all the differences were significant, most highly so. The responses to live or dead females were in accord with earlier conclusions except that results with living females were influenced by the females' unreceptive behavior. Young females were much less resistant to copulation and, up to 2 days, living females were successfully mated by a greater proportion of the males, the difference being highly significant in

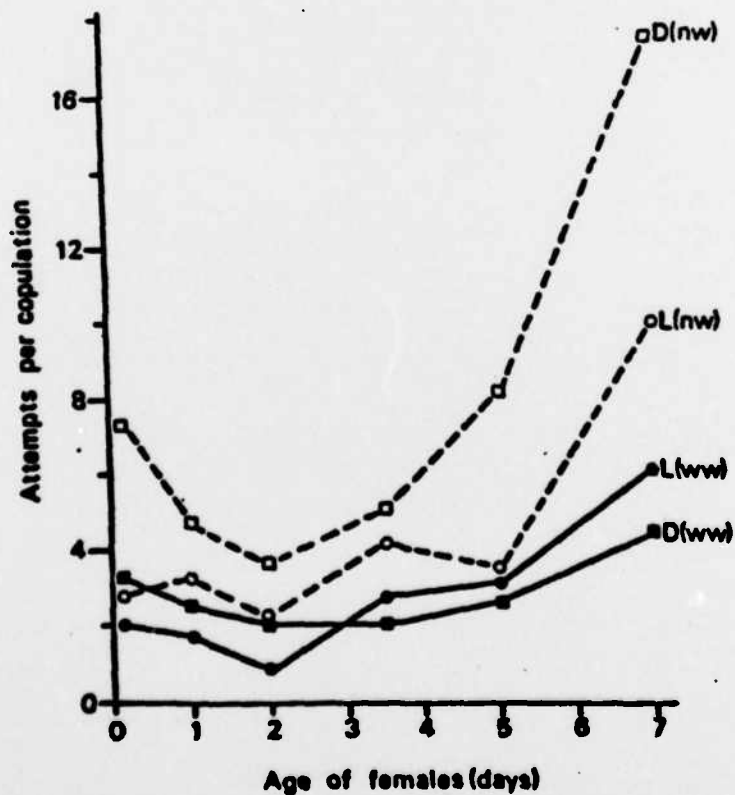
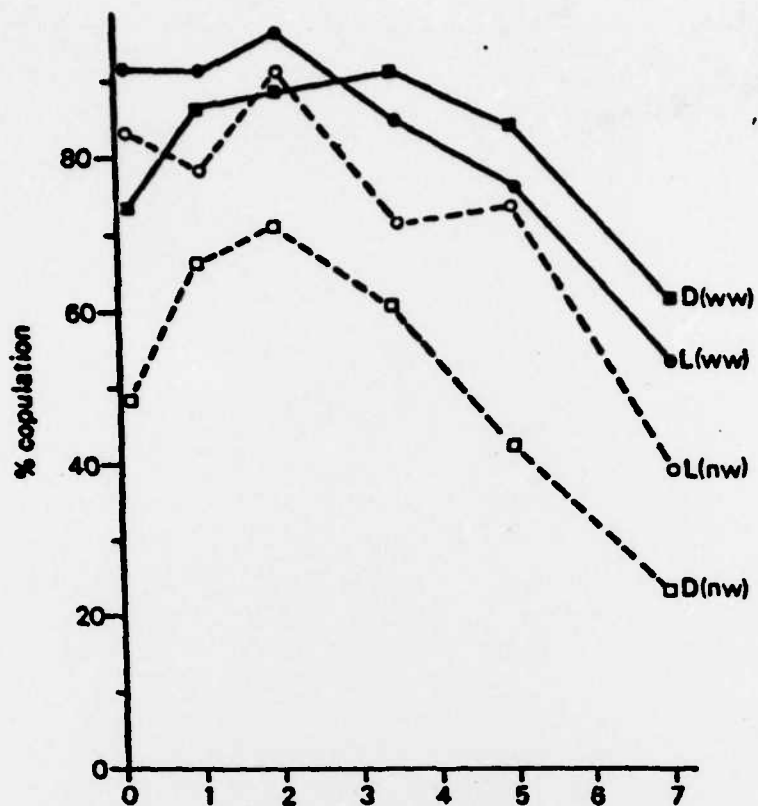


Fig. 4. Relationship of proportion of copulations achieved and number of male attempts per copulation to female age. L, live; D, dead; ww, with wings, nw, no wings.

TABLE 2. Proportion of females copulated as related to presence or absence of wings and living or dead state of females.

	Age of females (days)					
	1-2.(hr)	1	2	3.5	5	7
<u>Live ♀♀</u>						
Difference in Z (with wings - no wings)	8.3	13.3	5.0	13.3	3.3	15.0
χ^2	†	†	†	3.142	0.178	2.719
<u>Dead ♀♀</u>						
Difference in Z (with wings - no wings)	25.0	20.0	16.7	30.0	42.2	37.5
χ^2	7.869 ⁺⁺⁺	6.708 ⁺⁺⁺	5.208 ⁺	15.093 ⁺⁺⁺⁺	24.706 ⁺⁺⁺⁺	17.508 ⁺⁺⁺⁺
<u>With wings</u>						
Difference in Z (live ♀♀ - dead ♀♀)	18.3	5.0	8.3	-6.7	-8.2	-8.3
χ^2	6.984 ⁺⁺⁺	†	†	†	1.364	0.853
<u>No wings</u>						
Difference in Z (live ♀♀ - dead ♀♀)	25.0	11.7	20.0	10.0	30.7	14.1
χ^2	16.338 ⁺⁺⁺⁺	2.048	8.015 ⁺⁺⁺	1.350	11.699 ⁺⁺⁺⁺	2.843
† P < 0.05	++ P < 0.02		+++ P < 0.01		++++ P < 0.001	

† Expected values < 10

the youngest group (1-2 h, Table 2). At 3.5-7 days, as the females became increasingly unreceptive, males completed more copulations with dead females, although the differences were not significant. The same effect was apparent in the data assessed in terms of the number of attempts per copulation achieved (Fig. 4, lower). When no wings were present, a condition which itself reduces male response, live females were mated by a greater proportion of males in all groups, with the difference highly significant in 3 (Table 2). The effect of female unreceptivity did not hinder copulation as much as a lifeless female lacking wings, a combination of conditions which both reduce male response. In general, (Fig. 4, Table 2) the condition of female winglessness diminished male response more than the condition of female death.

V. DISCUSSION

It is very probable that sexual activity in C. melleus takes place very shortly after emergence as the adults walk over the surface of the breeding site (Linley & Adams 1972, 1974, Hinds & Linley 1974). Present results are generally in accord with this interpretation inasmuch that females are maximally stimulating to males over the first 2 days after emergence. However, an increase in stimulus with female age up to 2 days (and little decline by 3.5 days) is consistently shown in all experimental groups (Figs. 3, 4). These increases are found in most cases to be highly significant when data for females 1-2 h old are compared with those 2 days old (Table 3), and in all instances the proportions at 2 days are greater than at 1-2 h.

An explanation of this takes into account other known attributes of the behavior. Linley & Mook (1975) have shown that sexually

TABLE 3. Comparisons of proportions of females copulated and male responses in categories 1 + 2, between females 1-2 hr old and 2 days old.

Condition of females	χ^2 for proportions of responses 1 + 2	χ^2 for proportions of copulations achieved
L(wv)	5.683 ⁺⁺	†
L(nw)	9.809 ⁺⁺⁺	1.905
D(wv)	8.875 ⁺⁺⁺	4.357 ⁺
D(nw)	9.202 ⁺⁺⁺	6.806 ⁺⁺⁺

⁺P < 0.05

⁺⁺P < 0.02

⁺⁺⁺P < 0.01

†Expected values < 5

experienced male and female C. melleus may interact behaviorally in such a way as to reduce the likelihood of an unreceptive female copulating with a male who has recently completed a mating. Females become unreceptive immediately after they mate, but virgin females also become progressively unreceptive as they age (earlier discussion). When it takes place between unreceptive virgins and recently mated males, who are seminally depleted (Linley & Hinds 1974), the interaction functions to reduce the probability of a virgin being inadequately inseminated (for details, see Linley & Mook 1975). Unfortunately, although the virgin male lacks the specific (probably mechanical) release response, which may often cause a non-virgin male to release an unreceptive female, the nature of the female's behavior is such that virgin males would also be less successful if some compensating factor did not exist. The increase in stimulating capacity of the female over the first 2 days almost certainly represents this factor. It is an adaptation to compensate for increasing unreceptivity in virgin females as they grow older. The effect is discernible in Fig. 4 and Table 3. In dead females, where unreceptive behavior did not offset the increase in female stimulating capacity, a significantly higher proportion of copulations was achieved at 2 days in both groups (Table 3). However, a significantly higher proportion of copulations was not achieved in live females, who resisted copulation to some degree. The proportion was higher at 2 days in each group, but not significantly so. After 2 or 3 days, it is assumed that almost all females will have mated, so the importance of maintaining or further increasing the stimulus experienced by males is reduced, as indicated in the data for days 4 through 7.

The stimulus which triggers male response is a contact pheromone (Linley & Carlson 1978), which is reasonably assumed to be relatively non-volatile. These data suggest that it is secreted by females up to about 3 days after emergence and after that at a greatly reduced level, or not at all. Slow evaporative loss of pheromone presumably accounts for the diminishing male response to progressively older females. Lang (1977) made similar observations in the mosquito Culiseta inornata. Female leg sets, which were initially highly potent in releasing copulation attempts by males, gradually lost this ability over a period of about 60 days.

In C. melleus, the legs, although not tested, may be important, but only at first contact, because males instantly clamber on top of females or seize their abdomens. The wings contribute substantially to the stimulus perceived by males and again, this contribution is probably in the early stages of male response and in the preliminary maneuvers of orientation.

We suggest that reduced male response to dead females may be connected with the number of tarsal contacts. Very young virgin C. melleus females are receptive to copulation, inasmuch as they offer relatively little resistance to the male. Even when 0-4 h old, however, only 16 of 40 virgin females showed no resistance at all (Linley & Adams 1974) and many kicked slightly at the male as he attempted to complete sexual union. Older females offered considerable resistance (Linley & Adams 1974). Considerably more manipulation with the legs and tarsal contact probably attends attempted copulation with a live female than with a dead one.

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